FEBS 28998 FEBS Letters 577 (2004) 551–554

# The role of bestrophin in airway epithelial ion transport

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Received 18 August 2004; revised 20 October 2004; accepted 21 October 2004

Available online 4 November 2004

Edited by Maurice Montal

**Abstract The purpose of this study was to identify $Cl^-$ channels in the basolateral membrane of airway epithelial cells at the molecular level. We have focused on a new family of $Cl^-$ channels, bestrophins, which have previously been identified in retinal pigment epithelium. RT-PCR, Western blot and confocal microscopy studies revealed the presence of bestrophin in airway epithelial cells. Decreasing bestrophin expression using siRNA resulted in diminished $^{36}Cl^-$ flux. These studies also showed that bestrophin regulation is similar to that of native basolateral $Cl^-$ channels. The data indicate that the presence of a functional bestrophin may contribute to the basolateral cell conductance in airway epithelial cells.**


*Keywords:* Small interfering RNA; Basolateral $Cl^-$ channel; cGMP

## 1. Introduction

The presence of $Cl^-$ channels in the basolateral membrane of airway epithelial cells has been established in several studies [1–4], but their physiological role is not well characterized. In a search for the molecular identity of the basolateral $Cl^-$ channels, we have considered the members of the well established (CLC, CFTR, and GABA/glycine receptors), and less well-characterized (CLIC or CLCA) $Cl^-$ channel families as possible candidates (for review see [5]). Among these, ClC-2 channels have been localized to the basolateral membrane in different cell types [6–8], but these channels are unlikely to play a major role in human airway epithelia because basolateral $Cl^-$ channels are regulated via cAMP-dependent pathways [3,9], while ClC-2 channels are not [5].

Recently, a new family of putative $Cl^-$ channels, bestrophins, has been identified in retinal pigment epithelium (RPE) [10]. Bestrophin is a protein encoded by the vitelliform macular dystrophy type 2 (VMD2) gene [11,12], which is mutated in Best macular dystrophy [13]. It localizes to the basolateral membrane of RPE cells [14] and constitutes a new family of $Cl^-$ channels unrelated in primary sequence to any previously characterized channel proteins [15–17].

In this study, we have investigated bestrophin expression in Calu-3 cells, an airway serous cell model [18], and used a small interfering RNA (siRNA) approach to transiently and specifically reduce its expression. Our findings indicate that bestrophin is present in Calu-3 cells and that ablation of its expression results in diminished apical-to-basolateral $^{36}Cl^-$ flux.

## 2. Materials and methods

### 2.1. Cell culture

Calu-3 cells were grown as previously described [9]. For transepithelial measurements, cells were seeded onto Costar Snapwell inserts (0.45-μm pore size) and grown submerged in culture medium for the first 6 days. Subsequently, air interface culturing was used, in which the medium was added only to the basolateral side of the inserts. Inserts were used for experiments 10–16 days after the establishment of an air interface.

### 2.2. RT-PCR

Forward and reverse primers for bestrophin (VMD2, GenBankTM Accession No. NM_0004183) were nucleotides 329–348 and 516–535, respectively. As a positive control, plasmid containing VMD2 gene (a generous gift from Dr. I. McDonald, Ophthalmology, University of Alberta) was used. DNA amplification was obtained by annealing for 1 min at 58 °C, followed by an elongation step at 72 °C for 1 min. The size of the amplified product was 207 bp and was sequenced by the University of Alberta DNA Sequencing Core Facility to confirm its identity.

### 2.3. Immunocytochemistry

Western blotting was performed as described elsewhere [19]. Primary rabbit polyclonal antibody against bestrophin (Bst-101AP) was purchased from FabGennix Inc., Shreveport, LA. Where appropriate, pre-absorption controls were carried out, using the blocking peptide supplied with the antibody. Controls were also carried out in which the primary antibody was replaced with PBS; these were negative (not shown). A minimum of three runs was carried out and a representative example is shown. Optical band densities were determined using Sigma Gel software (Jandel Scientific).

### 2.4. Transepithelial measurements

Ussing chamber studies were performed as previously described [20]. Cells grown on inserts were mounted into CHM5 Ussing chambers (WPI, Sarasota, Fl), and bathed on apical and basolateral sides with 10 ml of Krebs Henseleit Solution (KHS) containing (mM): 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 $KH_2PO_4$, and 11.1 glucose, pH 7.4 [9]. Solutions were warmed to 37 °C and circulated with a gas lift using 95% $O_2$–5% $CO_2$. During all experiments, the $I_{sc}$ was allowed to stabilize for 20 min prior to treatments. In all experiments, amiloride (10 μM, apical) was present in the apical compartment to inhibit ENaC-mediated $Na^+$ absorption. Stock solutions for 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, 5 mM), *S*-nitroso-glutathione (GSNO, 100 mM) and amiloride (10 mM) were prepared in $H_2O$, whereas forskolin (10 mM) was prepared in ethanol. All chemicals were from Sigma (St. Louis, MO).

### 2.5. RNA interference

The selection of the coding sequence for siRNA was determined using Qiagen siRNA Designer (Qiagen, Mississauga, Ont., Canada) and was analyzed by BLAST research to ensure that it did not have

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0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.10.068

significant sequence homology with other genes. The 21-oligonucleotides purchased as ready-annealed purified duplexes from Qiagen (sense 5′-CACAAGCAGUUGGAGAAACdTdT-3′, antisense 3′-dTdTGUGUUCGUCAACCUCUUUG-5′) targeted bestrophin mRNA sequence in positions 588–608. Transfection was carried out using Qiagen RNAi Starter Kit. Control siRNA was fluorescein-labeled, 21 bp of random sequences provided by Qiagen, and were also used to optimize transfection efficiency. In preliminary studies, we found that the 1:9 (μg:μl) ratio of RNAiFect Transfection Reagent to siRNA, produced the best results, with over 80% of cells being transfected. The experiments were performed with cells grown to confluency at the air–liquid interface. 14.5 μl of 20 μM siRNA and 33.75 μl of transfection reagent were added to a medium in a total volume of 250 μl, and left for 15 min at room temperature to allow formation of transfection complexes. Subsequently, the mixture was diluted with a complete cell culture medium to a total volume of 1 ml, added to the basolateral side, and the cells were incubated for 72 h.

### 2.6. Radioisotopic flux

Calu-3 inserts were short-circuited for 20 min prior to the addition of the radioisotope. At time zero ($T_0$), background samples were taken, followed by the addition of 3 μCi of $^{36}Cl^-$ (specific activity of 0.126 mCi/ml, Amersham Pharmacia Biotech, UK) to the basolateral compartment,and another 20 min allowed for the establishment of equilibrium. At this time ($T_{20}$), 0.5 ml of samples was taken from the apical side and replaced with fresh KHS; this was repeated at 10 min. intervals thereafter. Three samples were taken ($T_{20}$–$T_{40}$) before the addition of forskolin (10 μM); this was followed by two more samples taken ($T_{50}$ and $T_{60}$) before the addition of basolateral DIDS (50 μM) and a further two samples taken ($T_{70}$ and $T_{80}$) after DIDS treatment. Two samples were also taken from the basolateral side before treatment with forskolin, to calculate the specific activity. Samples were counted in a liquid scintillation counter and the basolateral-to-apical flux ($J^{Cl}_{BA}$) was calculated according to standard equations [21]. $^{36}Cl^-$ fluxesin the apical-to-basolateral ($J^{Cl}_{AB}$) direction were measured in exactly the same fashion, except that the radioisotope was added to the apical bathing solution. Net $^{36}Cl^-$ flux ($J^{Cl}_{net}$) was calculated as $J^{Cl}_{net} = J^{Cl}_{BA} - J^{Cl}_{AB}$.

### 2.7. Statistical analysis

Results are presented as means ± S.E.; $n$ refers to the number of experiments. The paired Student's $t$ test was used to compare the means of two groups. Differences among the means of multiple groups were determined by one-way analysis of variance with the Tukey–Kramer post-test using Instat 3.05 (Graphpad software, San Diego, CA).

## 3. Results

Fig. 1 shows that Calu-3 cells express bestrophin mRNA under normal cell culture conditions. To estimate the possible sensitivity of Calu-3 cells to bestrophin knockdown by siRNA, initial experiments were performed at 24 h post-transfection. No significant differences were revealed between control cells and cells transfected with bestrophin siRNA (data not shown). However, 72 h incubation with bestrophin-directed siRNA resulted in significant and specific suppression of bestrophin protein, as judged by Western blotting (Fig. 2). Measurement of band optical density indicated that the siRNA treatment reduced the bestrophin band intensity to 34.6 ± 5.8% ($n = 3$) of its control value.

The next set of experiments studied $I_{sc}$ in Calu-3 monolayers transfected with either bestrophin siRNA or with control siRNA (Fig. 3). The average transepithelial resistance and baseline $I_{sc}$ in bestrophin siRNA treated cell inserts were 356.5 ± 40 Ω cm$^2$ and 30.9 ± 2.7 μA cm$^2$ ($n = 6$), respectively, and were not different from control values (287.0 ± 24.5 Ω cm$^2$ and 41.6 ± 7.3 μA cm$^2$ ($n = 6$), respectively). Similarly, the effects of the nitric oxide donor, GSNO (100 μM), on the peak

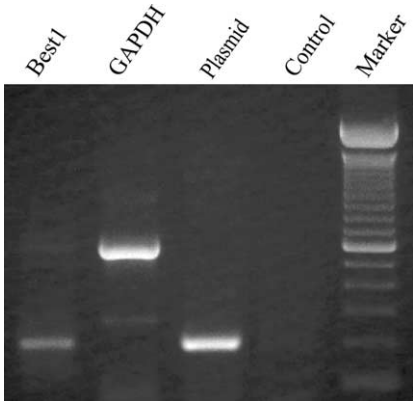


Fig. 1. Expression of bestrophin in Calu-3 cells. Calu-3 mRNA was reverse-transcribed into cDNA and PCR-amplified using bestrophin-specific primers (lane 1 from left). GAPDH amplification of the same sample is shown as a control for RNA quality. Plasmid containing VMD2 gene was used as a positive control. PCR amplification was also conducted on RNA preparations (without reverse transcription) to control for possible genomic contamination (lane 4). The expected sizes of the PCR products are 207 bp (bestrophin) and 595 bp (GAPDH). Marker-DNA standard (a 100-bp ladder).

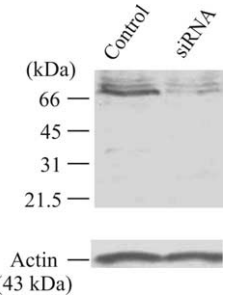


Fig. 2. Specific knockdown of bestrophin expression in Calu-3 cells by siRNA. Cells, cultured on 6-well plates, were subjected to transfection with either bestrophin siRNA or control siRNA. At 72 h post-transfection, cells were homogenized and representative samples were assayed by Western blotting. The blot shown is representative of three experiments and actin served as a loading control. The bestrophin antibody labels a 68-kDa protein in Calu-3 cells.

$I_{sc}$ were not significantly different in control and siRNA treated cells (41.1 ± 10.3 μA cm$^2$, $n = 3$ and 46.6 ± 6.6 μA cm$^2$, $n = 6$, respectively). However, subsequent application of DIDS (50 μM, basolateral) increased Isc by 25.6 ± 3.5 μA cm$^2$ ($n = 3$) under control conditions, but only by 7.4 ± 1.1 μA cm$^2$ ($n = 6$, $P < 0.01$) after siRNA treatment. These results suggest that bestrophin siRNA affects the function of basolateral DIDS-sensitive $Cl^-$ channels.

A direct way to show that bestrophin siRNA treatment affects chloride secretion is to measure chloride movement using $^{36}Cl^-$. Unidirectional $^{36}Cl^-$ fluxes were measured in both the basolateral to apical ($J_{BA}$) and in the apical to basolateral ($J_{AB}$) directions. Fig. 4 shows the net $^{36}Cl^-$ flux rate ($J^{Cl}_{net}$) during each sample interval in control and siRNA-treated cells. This information is supplemented with unilateral $^{36}Cl^-$ flux data in Table 1. Consistent with the findings from earlier studies [9,22], the data confirm that under basal conditions there is no net movement of $^{36}Cl^-$ in Calu-3 cells. Interestingly, cells treated with bestrophin siRNA showed higher baseline and forskolin

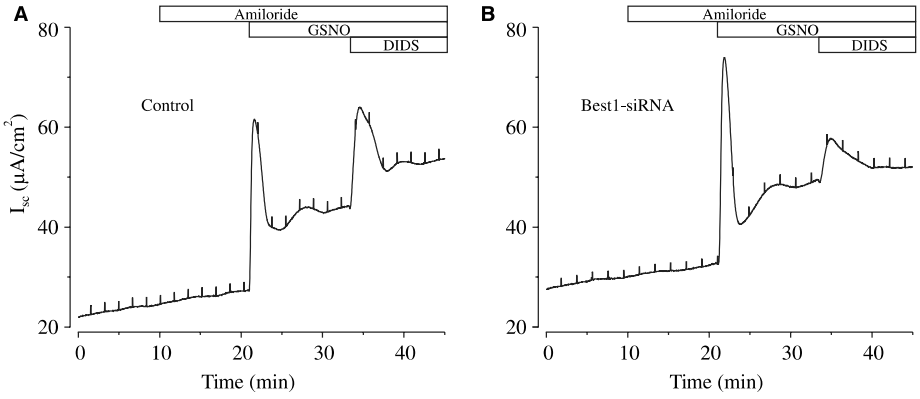


Fig. 3. Bestrophin siRNA reduces DIDS-sensitive basolateral $Cl^-$ conductance in Calu-3 cells. Representative recordings (siRNA-treated, $n = 6$; control, $n = 3$) show $I_{sc}$ responses to GSNO (100 μM, bilateral) and DIDS (50 μM, basolateral). The effect of DIDS on GSNO-activated $I_{sc}$ is reduced by more than 70% (from $14.8 \pm 1.3$ to $4.2 \pm 0.8$ μA/cm$^2$, $n = 6$) in bestrophin vs. control siRNA treated cells.

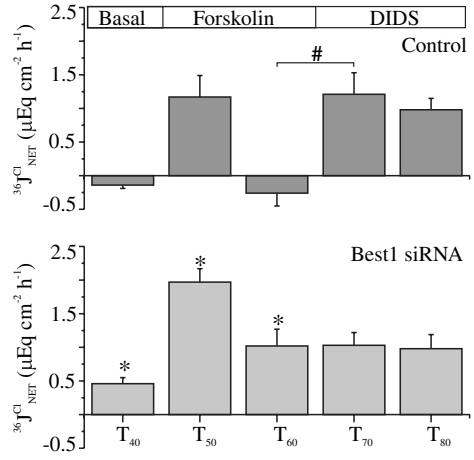


Fig. 4. Net $^{36}Cl^-$ fluxes in control and bestrophin siRNA-treated cells. Measurements were performed under basal conditions ($T_{40}$), in the presence of 10 μM forskolin ($T_{50}$ and $T_{60}$) and 50 μM DIDS ($T_{70}$ and $T_{80}$). Net fluxes were obtained from six experiments. $^*P < 0.05$, fluxes in control and siRNA-treated cells. $^\#P < 0.01$, effect of DIDS under control conditions.

stimulated net $^{36}Cl^-$ fluxes than control cells (Table 1, $T_{50}$ and $T_{60}$). Subsequent application of DIDS (50 μM, basolateral) decreased $J_{AB}$ from $2.92 \pm 0.30$ to $1.30 \pm 0.46$ μEq cm$^{-2}$ h$^{-1}$ ($P < 0.05$) under control conditions, but had no significant effect on $^{36}Cl^-$ flux in siRNA treated cells (Table 1, $T_{70}$ and $T_{80}$).

## 4. Discussion

Since its discovery in *Caenorhabditis elegans* [23], RNA interference has become an effective method for the analysis of gene function [24]. We used this technique to study the bestrophin gene–function relationship in human airway epithelial cells. Our results suggest that bestrophin functions as a basolateral $Cl^-$ channel regulated via cAMP- and cGMP-dependent phosphorylation.

Current models of bestrophin topology suggest that this protein consists of either four [10,25] or six [16,17] transmembrane spanning α-helices and a large C-terminal cytoplasmic region, containing four potential phosphorylation sites for protein kinase A, two for protein kinase C, and three for cGMP-dependent protein kinase (PKG) [26]. In earlier studies, we had shown that GSNO stimulates transepithelial anion secretion acting via a cGMP-dependent pathway [20]. More recently, basolateral $Cl^-$ channels were shown to be activated via cAMP-dependent phosphorylation [9]. The results of this study show that silencing the bestrophin gene with siRNA decreases GSNO-activated, DIDS-sensitive $I_{sc}$, and forskolin-activated $^{36}Cl^-$ flux. This suggests that bestrophin functions as a basolateral $Cl^-$ channel regulated via cAMP- or cGMP-dependent phosphorylation. There is also the possibility of cross talk between cAMP- and cGMP-dependent pathways because cAMP inhibits cGMP-specific phosphodiesterase 5, and both cAMP and cGMP can activate PKG [27]. Bestrophin is known to interact with protein phosphatase 2A [28] and phosphorylation/dephosphorylation of its C-terminal could act as the on/off switch regulating channel activity.

Table 1
Unidirectional and net ion fluxes across short-circuited Calu-3 monolayers

| Sample | Treatment | Control | | | Bestrophin siRNA-treated | | |
|---|---|---|---|---|---|---|---|
| | | $J_{BA}$ | $J_{AB}$ | $J_{NET}$ | $J_{BA}$ | $J_{AB}$ | $J_{NET}$ |
| $T_{40}$ | Baseline | $0.98 \pm 0.21$ | $1.11 \pm 0.11$ | $-0.13 \pm 0.18$ | $1.41 \pm 0.23$ | $0.95 \pm 0.08$ | $0.46 \pm 0.09^*$ |
| $T_{50}$ | Forskolin | $3.57 \pm 0.60$ | $2.40 \pm 0.23$ | $1.17 \pm 0.32$ | $4.46 \pm 0.38$ | $2.48 \pm 0.23$ | $1.97 \pm 0.19^*$ |
| $T_{60}$ | Forskolin | $2.67 \pm 0.23$ | $2.92 \pm 0.30$ | $-0.26 \pm 0.19$ | $3.18 \pm 0.31$ | $2.16 \pm 0.40$ | $1.02 \pm 0.16^*$ |
| $T_{70}$ | +DIDS | $2.51 \pm 0.44$ | $1.30 \pm 0.46$ | $1.21 \pm 0.32$ | $3.28 \pm 0.23$ | $2.25 \pm 0.32$ | $1.03 \pm 0.26$ |
| $T_{80}$ | +DIDS | $2.31 \pm 0.31$ | $1.33 \pm 0.14$ | $0.98 \pm 0.17$ | $2.80 \pm 0.26$ | $1.81 \pm 0.33$ | $0.98 \pm 0.21$ |

Flux values are in μEq cm$^{-2}$ h$^{-1}$. Forskolin (10 μM) was added bilaterally, DIDS (50 μM) was added basolaterally. Each unidirectional flux was obtained from $n = 3$ experiments, therefore net fluxes from $n = 6$ experiments.
$^*P < 0.05$, significantly different from the corresponding flux in control cells.

Western blot experiments suggest that siRNA treatment reduces bestrophin expression in Calu-3 cells by ~65%. However, measurements of $^{36}Cl^-$ flux indicate that bestrophin siRNA reduces basal $J_{BA}$ by only ~14%. This suggests that bestrophin may not be the only $Cl^-$ channel present in the basolateral membrane. There is significant evidence that ubiquitously expressed ClC-2 $Cl^-$ channels are present in the basolateral membrane of epithelial cells [6–8,29] and these channels could also contribute to the basolateral $Cl^-$ conductance.

Although bestrophins were initially shown to function as the $Cl^-$ channels in RPE cells [15,16], there is now significant evidence to suggest that these channels could also play an important physiological role in other tissues. Boese et al. [30] have shown that $Cl^-$ conductances in collecting duct cells have some important similarities to bestrophin-induced conductances [30]. Others have described cGMP-activated $Cl^-$ channels in smooth muscle cells that share some characteristics with the bestrophin channel family [31,32]. Interestingly, recent studies with a member of the CLCA channel family, pCLCA1, have found that although the CLCA protein localized to the basal RPE, its primary function was to regulate the activity of other proteins rather than to mediate $Cl^-$ conductance [33]. Although the results of our study strongly suggest that bestrophin functions as a basolateral $Cl^-$ channel, we cannot exclude the possibility that this protein may function as a regulatory component of an ion channel-protein complex. However, recent experiments showing that mutations of bestrophin residues in a putative channel pore alter the relative permeability and conductance for different anions [17] provide strong evidence that bestrophins function as ion channels, rather than regulators of channel activity.

In conclusion, the results of our studies show, for the first time, that bestrophin is present in airway epithelial cells and functions as a basolateral $Cl^-$ channel. We also show that bestrophin channel activity in Calu-3 cells is regulated via cAMP- and cGMP-dependent pathways. Basolateral $Cl^-$ channels have previously been proposed to play a crucial role in determining the magnitude of $Cl^-$ and $HCO_3^-$ secretion [9], and the knowledge of their molecular identity may have important clinical implications.

*Acknowledgements:* This work was supported by grants from the Canadian Institutes of Health Research and the Canadian Cystic Fibrosis Foundation.

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